Analysis of Non-Template-Directed Nucleotide Addition and Template Switching by DNA Polymerase†

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ABSTRACT: DNA polymerases use an uninterrupted template strand to direct synthesis of DNA. However, some DNA polymerases can synthesize DNA across two discontinuous templates by binding and juxtaposing them, resulting in synthesis across the junction. Primer/template duplexes with 3′ overhangs are especially efficient substrates, suggesting that DNA polymerases use the overhangs as regions of microhomology for template synapsis. The formation of these overhangs may be the result of non-template-directed nucleotide addition by DNA polymerases. To examine the relative magnitude and mechanism of template switching, we studied the in vitro enzyme kinetics of template switching and non-template-directed nucleotide addition by the 3′-5′ exonuclease-deficient large fragment of *Escherichia coli* DNA polymerase I. Non-template-directed nucleotide addition and template switching were compared to that of standard primer extension. We found that non-template-directed nucleotide addition and template switching showed similar rates and were approximately 100-fold slower than normal template-directed DNA synthesis. Furthermore, non-template-directed nucleotide addition showed a 10-fold preference for adding dAMP to the ends of DNA over that of the other three nucleotides. For template switching, kinetic analysis revealed that the two template substrates acted as a random bireactant system with mixed-type inhibition of substrate binding by one substrate over the other. These data are the first to establish the binding kinetics of two discontinuous DNA substrates to a single DNA polymerase. Our results suggest that although the activities are relatively weak, non-template-directed nucleotide addition and template switching allow DNA polymerases to overcome breaks in the template strand in an error-prone manner.

DNA polymerases must faithfully duplicate the genome of an organism. However, all DNA polymerases introduce errors during the course of every round of replication. If not repaired, these errors can potentially result in mutation. The fidelity of DNA polymerases depends on several factors including the presence of proofreading activity, deoxynucleoside triphosphate pools, and the sequence and structure of the DNA template (*1*).

The presence of a strand break in the template presents an especially challenging substrate for DNA synthesis and fidelity. Normally, DNA synthesis stops when a DNA polymerase reaches either a strand break or the end of the template strand. However, a number of DNA polymerases, most notably those lacking 3′-5′ exonuclease activity, can synthesize DNA in a non-template-directed manner, resulting in one or more nucleotide additions to the end of the nascent strand. For example, the exonuclease-free derivative of the large fragment of *Escherichia coli* DNA polymerase I (exo$^-$ KF)[1] synthesizes at least one nucleotide in a non-template-directed manner (*2*). The DNA polymerase of *Thermus aquaticus* also shows non-template-directed nucleotide addition. This activity has been exploited to form the basis of a popular cloning strategy (*3, 4*). Furthermore, purified eukaryotic DNA polymerase α also has this activity (*5*).

In addition to non-template-directed nucleotide addition, or perhaps because of it, many of the same DNA polymerases also show template-switching activity. For our purposes, template switching is broadly defined as the ability of a DNA polymerase to synthesize a nascent strand of DNA from two discontinuous template strands. The term was originally used to describe the products seen in DNA synthesized by *E. coli* DNA polymerase I (Pol I) in vitro (*6*). The reaction products showed very rapid renaturation kinetics after heat or alkali denaturation as well as branched structures under electron microscopy (*7, 8*). Kornberg and his colleagues proposed a mechanism in which Pol I synthesizes DNA on one strand of a duplex substrate while displacing the non-template strand. Pol I then releases the template strand and binds the displaced strand before its 5′-3′ exonuclease activity degrades it—in effect switching templates. The resulting products from such a template-switching event eventually lead to hairpin structures that explain the rapid renaturation kinetics and branched structures seen in electron micrographs.

More recently, template switching describes the phenomenon whereby exo$^-$ KF synthesizes DNA across discontinuous templates. Clark demonstrated that exo$^-$ KF could perform DNA synthesis on a blunt-ended primer/template

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[1] Abbreviations: dNTPs, deoxynucleoside triphosphates; EDTA, ethylenediaminetetraacetic acid; Pol I, *E. coli* DNA polymerase I; KF, large (Klenow) fragment of Pol I; exo$^-$ KF, exonuclease-free KF; K_D^{app}, apparent dissociation constant; V_{max}, maximal velocity, k_{cat}, catalytic turnover; T, template strand; P:C, primer/complement duplex.

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duplex in the presence of an unlinked single-stranded oligonucleotide template (*9*). The unlinked single-stranded oligonucleotide determined the sequence of the added nucleotides. He proposed that template switching was due to the ability of exo$^-$ KF to juxtapose two unlinked templates through the use of small regions of complementarity at the 3′ ends of the primer and the unlinked template strand. This is most likely the result of a non-template-directed nucleotide addition of a single nucleotide to the 3′ end of the primer. King et al. confirmed and extended Clark's results by showing that template switching occurs on primer duplexes with a variety of blunt and protruding single strands through a so-called fill-in mechanism similar to that proposed by Clark (*10*, *11*). In this model, a 5′ overhang is filled in to form a blunt-ended intermediate that is converted to a single-nucleotide 3′ overhang intermediate through non-template-directed nucleotide addition. This overhang is used as a region of complementarity to juxtapose an unlinked template strand, and synthesis continues to the end of the newly aligned template strand. Additionally, they proposed an overlap mechanism in which a primer/template duplex with a 3′ overhang would base pair with an unlinked template strand through a 3′ overhang independent of non-template-directed nucleotide addition. In this case, exo$^-$ KF functions to align and join the two discontinuous DNA substrates. Like non-template-directed nucleotide addition, template switching is not limited to exo$^-$ KF. It has been observed with Taq DNA polymerase, as well as purified human DNA polymerase α, and there is even evidence that human DNA polymerases β, δ, and ϵ are capable of small amounts of template switching (*12*).

What is not clear from the previous studies is the relative magnitude, rate, and mechanism of non-template-directed nucleotide addition and template switching. For example, both Clark and King et al. used nearly stoichiometric ratios of duplex substrate to enzyme (1:5.7 and 7.1:1, respectively) for their template-switching studies. At high enzyme/substrate ratios calculating the catalytic turnover for the reactions becomes problematic. In an effort to understand the mechanisms of non-template-directed nucleotide addition and template switching, we characterized the kinetic parameters of these activities for exo$^-$ KF by examining the individual rates of non-template-directed nucleotide addition and template switching as compared to normal template-directed primer extension. We report here that template switching and non-template-directed nucleotide addition both approximate classical Michaelis−Menten kinetics at catalytic levels of exo$^-$ KF, that their rates appear to be comparable to one another, and that their rates are about 100-fold slower than normal primer extension measured on a continuous template. Mechanistically, both discontinuous substrates bind the enzyme in a random sequential order with each substrate affecting the binding of the other.

MATERIALS AND METHODS

Materials. [γ-^{32}P]-ATP (3000 Ci/mmol) was purchased from Amersham Biosciences (Arlington Heights, IL). T4 polynucleotide kinase and deoxynucleoside triphosphates were obtained from Roche Applied Science (Indianapolis, IN). *E. coli* exo$^-$ KF fragment was obtained from New England BioLabs (Beverly, MA). HPLC-purified oligonucleotides were purchased from Qiagen (Alameda, CA). All other chemicals were obtained from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ).

Template-Switching Assay. The assay to measure template switching has been described elsewhere (*12*). Briefly, the assay uses a double-stranded primer/template duplex either radiolabeled or Cy-5 labeled at the 5′ end of the primer strand. Annealing the complementary template strand, 5′-CCAGTCCGGGAACAA-3′, to the labeled primer strand, 5′-TTGTTCCCGGACTGGTAT-3′, generates a duplex with a 3-nucleotide-long 3′ overhang on the primer strand. Primer extension proceeds when exo$^-$ KF juxtaposes the duplex substrate and an unlinked template strand, 5′-CGTAAC-TATGCGGCATCAGAGCAGATTGATA-3′, using the overhang on the primer as a source of complementarity to the 3′ end of the unlinked template. The free 3′-hydroxyl group on the primer strand serves as the site of DNA synthesis. Product formation was determined electrophoretically as an increase in the length of the labeled primer strand (see below). All assays were carried out in the presence of 25 mM Tris-HCl (pH 7.2), 0.2 mM dNTPs, 5 mM $MgCl_2$, 5 mM dithiothreitol, and 0.2 mg/mL bovine serum albumin at 37 °C unless stated otherwise. Reaction products were measured at 10, 20, 40, and 60 min. The final enzyme concentration for these experiments and for the non-template-directed nucleotide addition assays was 73.5 fM.

Primer-Extension Assay. All template-switching experiments were compared to results from a gel-based primer-extension assay. The assay conditions were identical to those used in the template-switching assay except for the following: substrate, time points taken, and enzyme concentration. The assay uses a substrate where the primer strand (identical to that used in the template-switching assay) is annealed to a long complementary template strand (Figure 1B), essentially mimicking the intermediate formed in the template-switching reaction after template synapsis (Figure 1A). The sequence of the long template strand in the primer extension assay is a composite of the complement strand and the template strand in the template-switching assay. The reaction products were measured at 5, 10, and 15 min. The final enzyme concentration was 7.35 fM.

Non-Template-Directed Nucleotide Addition Assay. Non-template-directed nucleotide addition was measured using the labeled primer strand of the template switching and primer-extension assays annealed to an oligonucleotide, 5′-ATACCAGTCCGGGAACAA-3′, generating a blunt end duplex substrate. The assays were run under similar conditions as before, except that the substrate was kept constant and a time course was taken.

For the experiments measuring kinetic parameters, individual dNTPs or a mix of the four nucleoside triphosphates was added in a range of concentrations from 0.01 μM to 1 mM. A saturating concentration, typically 50 μM, of the P:C substrate in Figure 1D was used, and the reactions were carried out as before for 20 and 40 min. Higher concentrations (100 μM) of the P:C substrate showed similar rates of non-template-directed nucleotide addition in the presence of all four nucleotides to that seen at 50 μM verifying that the P:C substrate concentration used was saturating.

Electrophoresis. Denaturing electrophoresis-loading dye (80% [v/v] formamide, 10 mM NaOH, 1 mM EDTA, 0.1% [w/v] bromophenol blue, 0.1% [w/v] xylene cyanol, 0.1%

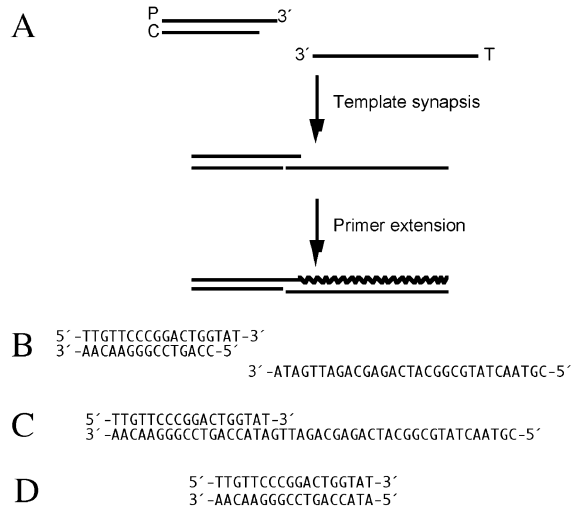

FIGURE 1: General features of the template-switching assay and substrates used. (A) A schematic representation of the two major steps in template switching. First, template synapsis occurs; DNA polymerase juxtaposes two discontinuous templates. One template (labeled "C") is complementary to the primer (labeled "P") forming a duplex with a 3-nucleotide-long single-stranded region on the primer strand (3′ overhang). The other template is single-stranded (to allow for complete extension) with three nucleotides at its 3′ terminus complementary to the 3′ overhang on the P strand. This short stretch of microhomology is used by the polymerase presumably to stabilize the T strand to the P strand. After template synapsis, the DNA polymerase can extend the primer in a manner directed by the T template strand (primer extension). The primer is 5′ end-labeled with either ^{32}P or Cy-5. (B) The sequences of the substrates used in the template-switching assay. (C) The sequence of the substrate used in the primer extension assay. The assay simply measures extension of the labeled P strand. The substrate mimics the reaction intermediate in template switching after template synapsis has occurred. (D) The sequence of the substrate used to measure non-template-directed nucleotide addition. A blunt substrate is used and the extension of the P strand is measured.

[w/v] orange G) was added at each time point in a reaction except for Cy-5 labeled DNA where the bromophenol blue and xylene cyanol were omitted. The double-stranded DNA products were denatured by incubating at 95 °C for 10 min and were then quick-chilled on ice for several minutes.

Samples were applied to a 15% denaturing polyacrylamide-sequencing gel (19:1 acrylamide/bisacrylamide) containing TBE (89 mM Tris base − boric acid, 2 mM EDTA) and 7 M urea. The samples were subjected to electrophoresis at a voltage of 40 V/cm (60 W). After electrophoresis, the gels were dried and gels containing Cy-5 labeled DNA were visualized using the 460 nm laser on a Molecular Dynamics Storm 860 system. Dried gels with ^{32}P-labeled DNA were exposed to Phosphorimager screens for several hours and the screens were then scanned with the Storm 860 system. Data were quantified using the ImageQuant software supplied with the Phosphorimager.

Enzyme Kinetics. Steady-state product levels were measured at various times for each assay within the linear response of the reaction. All experiments were carried out at least 3 times taking 2−4 time points for each experiment depending on the linearity of the reaction. Every gel contained internal quantitative standards of known concentration and specific activity so that integrated densitometry values for each product band could be converted to known molar concentrations (expressed as nanomolar) for each reaction. Rates of reaction for template switching and primer extension were calculated and kinetic parameters were determined using standard reciprocal plot analysis (*13*).

RESULTS

Purified bacterial DNA polymerases lacking 3′-5′ exonuclease activity can bridge unlinked discontinuous template strands and initiate DNA synthesis across the joined strands, using either double-stranded or single-stranded oligonucleotides as model substrates for double-strand breaks (*9*, *10*). In an attempt to understand the mechanism of template switching, we chose to determine the kinetic parameters of template switching by exo$^-$ KF and compare them to those determined for a primer extension control assay. We also examined the relative magnitudes of template switching and non-template-directed nucleotide addition.

Template Switching. To measure template switching, we used a previously developed assay depicted in Figure 1A (*12*). Briefly, the assay uses two DNA substrates: a double-stranded primer/complement (P:C) duplex with a three-nucleotide-long 3′ overhang; and a single-stranded template (T) containing a three-nucleotide-long terminus complementary to the 3′ overhang on the P:C substrate (Figure 1B). It has been previously demonstrated that template switching works if both substrates are double stranded (*10*). However, as exo$^-$ KF has no 5′-3′ exonuclease activity and presumably no strand displacement activity, and since no helicases are present in the reaction, the single-stranded template facilitates primer extension analysis by allowing the visualization a longer product on the gel. The appropriate controls for the assay have been previously reported (*10*, *12*). For example, template switching does not occur with two terminally complementary single strands (i.e., a missing C strand). The 5′ end-labeled primer strand is 18 nucleotides long and the template strand is 31 nucleotides long (Figure 1B). Thus, if DNA polymerase facilitates their annealing (template synapsis) using the three-nucleotide-long region, and completely extends the primer strand using the newly synapsed template strand, a 46-nucleotide product will result. The assay therefore is a composite of at least two activities on the part of DNA polymerase: template synapsis and primer extension (which is itself composed of 29 polymerization steps).

To determine the kinetic values and to facilitate comparison with the primer extension assay (see below), reactions were carried out at various primer/complement substrate concentrations while template substrate concentrations remained constant. Figure 2A depicts a representative scanned gel showing the product formed after 10 min at concentrations of primer/complement substrate from 1 to 25 μM. The template substrate was kept constant (158 μM) and the [T]/[P:C] molar ratios ranged from 6.2 to 158. The product bands from the gel were scanned and integrated using internal standards in each gel (data not shown) to obtain the amount of product formed and the velocity of the reaction. Rates of reaction for a representative gel are plotted in Figure 3A. The results strongly suggest that the reaction behaved with Michaelis−Menten kinetics. Using reciprocal plot analysis, we calculated kinetic values for the reaction (Table 1) and determined the catalytic turnover number for the template switching activity of exo$^-$ KF to be 0.26 min^{-1}.

Note that we consistently observed fainter, more diffuse bands above the full-length product band (Figure 2B),

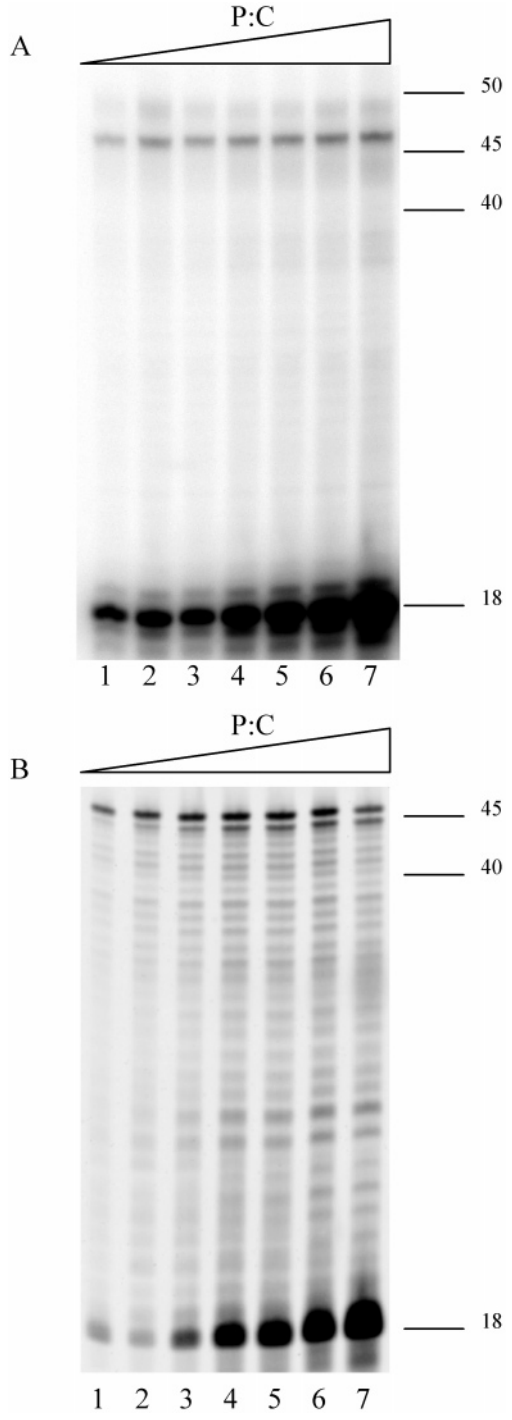

FIGURE 2: Two 15% denaturing polyacrylamide gels show template switching and primer extension. (A) Template switching was measured after 10 min. Each reaction has various concentrations of the P:C duplex substrate at constant T strand concentrations (see Table 1 for concentrations). The 5′-labeled L strand is 18 nucleotides long. If template switching occurs between the P:C substrate and the T strand, the P strand is extended 29 nucleotides resulting in a 46-nucleotide-long product (molecular weight markers were run in a companion lane; not shown). Note that in addition to incomplete primer extension products, we routinely saw products that were larger than the full-length product suggesting that non-template-directed nucleotide addition was occurring. (B) Primer extension after 10 min at various concentrations of the primer extension substrate depicted in Figure 1C. The full-length product is also 46 nucleotides long. A significant amount of incomplete primer extension was observed in both the primer extension and template switching experiments. However, only full-length products were used in determining the rates of reaction.

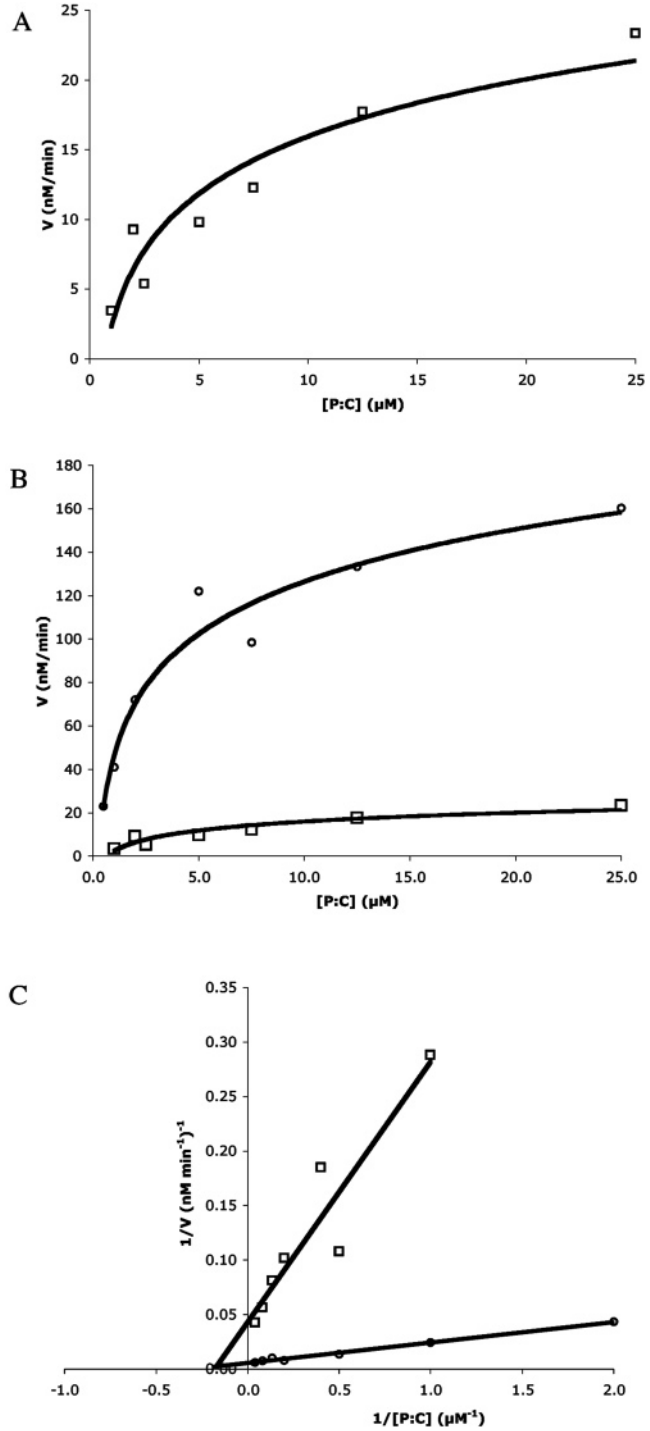

FIGURE 3: Rates of reaction at various duplex substrate concentrations (P:C) for (A) template switching (open squares); and (B) primer extension (open circles) both under saturating template concentrations. The data for template switching in panel A is replotted against the data for primer extension for comparative purposes. (C) Reciprocal plots for template switching (open squares) and primer extension (open circles). The complete data is presented in Table 1.

suggesting that products were formed that were longer than the expected 46-nucleotide product. These observations suggest that after the DNA polymerase has fully extended the primer to the end of the synapsed template, it adds one or more nucleotides to the end of the product in a non-template-directed manner. These bands were included in the template-switching analysis. They were also used to deter-

Table 1: Kinetic Values for Template Switching and Primer Extension

[P:C] (μM)	[T][a] (μM)	n	v (nM min^{-1}) ± SEM	K_D^{app} (μM) ± SEM	V_{max}^{app} (nM min^{-1}) ± SEM	K_D^{app}/V_{max}^{app}	k_{cat} (min^{-1})	k_{cat}/K_D^{app} (μM^{-1} min^{-1})
				Template Switching				
1.00	158	12	1.41 ± 0.45	12.3 ± 1.43	18.9 ± 4.01	0.65	0.26	0.02
2.00	158	12	3.64 ± 1.23					
2.50	158	12	2.62 ± 0.74					
5.00	158	12	5.01 ± 1.15					
7.50	158	12	6.77 ± 1.58					
12.5	158	8	9.40 ± 3.36					
25.0	158	12	11.8 ± 3.09					
				Primer Extension				
0.50		9	34.6 ± 8.36	3.25 ± 0.57	211 ± 14.3	1.56×10^{-2}	28.7	8.83
1.00		9	62.3 ± 15.2					
2.00		9	102 ± 21.8					
5.00		9	145 ± 21.0					
7.50		9	130 ± 16.5					
12.5		9	155 ± 20.0					
25.0		3	108 ± 27.6					

[a] For template switching, the P:C substrate concentration was varied from between 1.0 μM to 25 μM at constant T substrate concentration. For primer extension, the discontinuous template strand was omitted; see Figure 1C.

Table 2: Kinetic Parameters for the P:C Substrate (A) under Various Constant T Concentrations and Replotted for T Substrate Parameters under Various Constant P:C Concentrations (B)

A

[T][a] (μM)	K_D^{app} [c] (μM) ± SEM	V_{max}^{app} (nM min^{-1}) ± SEM	K_D^{app}/V_{max}^{app}	k_{cat} (min^{-1})	k_{cat}/K_D^{app} (μM^{-1} min^{-1})	n
13.1	4.60 ± 0.23	2.39 ± 0.62	1.92	0.03	7.06×10^{-3}	5
26.3	4.73 ± 0.94	2.94 ± 0.70	1.61	0.04	8.46×10^{-3}	8
52.5	7.43 ± 1.16	8.26 ± 3.56	0.90	0.11	1.51×10^{-2}	6
105	10.7 ± 1.53	12.3 ± 3.25	1.13	0.13	1.20×10^{-2}	10
158	12.3 ± 1.43	18.9 ± 4.01	0.65	0.26	2.09×10^{-2}	12

B

[P:C] (μM)	K_D^{app} [c] (μM)	V_{max}^{app} (nM min^{-1})	K_D^{app}/V_{max}^{app}	k_{cat} (min^{-1})	k_{cat}/K_D^{app} (μM^{-1} min^{-1})
0.50	53.8	1.50	35.9	0.02	5.57×10^{-4}
1.00	55.6	1.70	32.7	0.02	6.12×10^{-4}
2.00	66.2	4.10	16.1	0.06	3.73×10^{-3}
2.50	75.2	5.10	14.7	0.07	4.76×10^{-3}
5.00	84.0	5.80	14.5	0.08	5.52×10^{-3}
7.50	84.0	6.90	12.2	0.09	7.38×10^{-3}
12.5	150	15.8	6.33	0.21	3.32×10^{-2}

mine the rates of non-template-directed nucleotide addition (see below).

To examine the mechanism of template switching, we conducted a series of kinetic experiments varying the concentration of the P:C substrate at various constant T substrate concentrations while keeping the dNTP concentration at saturating levels. The results (Table 2A) show that as the T substrate concentration increases the V_{max} of the reaction increases as well. This is to be expected since the reaction is dependent on the T substrate. Interestingly, the K_D^{app} for P:C binding also increases with increasing T concentrations, suggesting that T is acting as a mixed-type inhibitor for binding of the P:C substrate to the enzyme. If the data are replotted, varying the concentration of T for various constant P:C concentrations, the results obtained are similar (Table 2B). Increasing the P:C concentration increases the V_{max} of the reaction as well as the K_D^{app} for the T substrate. The reciprocal plots of the kinetic values for 1/[P:C] at various constant T concentrations (Figure 4A) and for 1/[T] at various constant P:C concentrations (Figure 4B) graphically demonstrate that the lines for both plots intersect below the 1/[substrate] axes and are indicative that both substrates act to inhibit the binding of the other. Furthermore, the two graphs are characteristic of two substrates in a randomly ordered sequential bireactant system (*13*). The *x*-value of the vertex for each plot is $-1/K_D$ for that particular substrate. For example, as [T] $\rightarrow$ 0 in Figure 4A, the theoretical reciprocal plot would become vertical and the K_D^{app} would reflect the true K_D for [P:C]. In this case, K_D = 3.69 μM for the P:C substrate. The *y*-value of the vertex was $1/V_{max}(1 - \alpha)$, where $\alpha = 3.69$, which yielded a V_{max} = 20.7 nM min^{-1}. Similar analysis was performed on the replotted data in Figure 4B: K_D = 45.5 μM for the T substrate and the V_{max} = 14.9 nM min^{-1} ($\alpha = 3.30$).

The results indicate that exo$^-$ KF binds two DNA substrates simultaneously and that each substrate has an effect on the binding of the other. Physical evidence for binding two discontinuous substrates can be found in the crystal structure of KF showing the binding of two discontinuous primer strands, apparently one at or near the 3′-5′ exonuclease active site (*14*). In our results, binding of the primer/complement duplex substrate is preferred over the single-stranded T substrate by nearly 14-fold. Since, exo$^-$ KF usually binds single-stranded DNA with higher affinity than

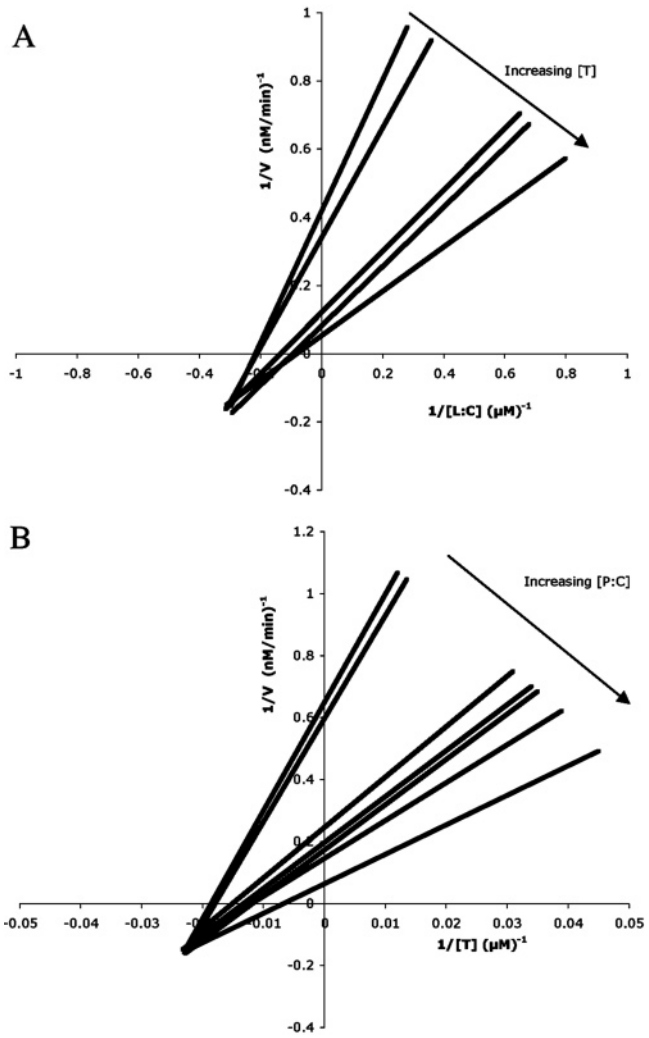

FIGURE 4: Reciprocal plots of template switching rates of reaction for varying concentrations of one substrate under various constant concentrations of the other substrate. (A) The rates of reaction were plotted as a function of P:C concentration (0.50−12.5 μM) for five different fixed T concentrations (ranging from 13.1 to 158 μM). (B) The rates of reaction were replotted as a function of T concentration for seven different fixed P:C concentrations. Arrowed line indicates the direction of the increasing constant substrate concentration. Data points were omitted.

double-stranded DNA (*6*), our results strongly suggest that in this case DNA polymerase recognizes the DNA terminus. The 3′ overhang is certainly critical for efficient template-switching activity (*11*, *12*); the maximal rates of template-switching activity is seen with three- and four-nucleotide-long overhangs (compared to blunt, 1-, and 2-nucleotide-long overhangs). However, no significant difference in K_D^{app} between a blunt end substrate versus a three-nucleotide-long 3′ overhang substrate was seen (Faucett and Islas, manuscript in preparation). Thus, the different rates seen in blunt versus overhang substrates is likely due to the time needed to create an overhang through non-template-directed nucleotide addition (see below).

Primer Extension. To compare the template-switching activity of exo$^-$ KF to its polymerization activity, we used an identical assay to the template-switching assay except that the substrate used had the primer annealed to one long continuous template strand (Figure 1C). The sequence of both the primer and template strands were identical to the substrates in the template-switching assay. In essence, the substrate mimics what is believed to be a reaction intermediate in template switching after template synapsis but before the DNA polymerase extends the primer.

The primer-extension experiments were carried out under identical conditions as that of the template-switching assays, except that a 10-fold lower concentration of enzyme was used (7.35 fM) and time points were taken sooner. A typical gel is depicted in Figure 2B. The concentration range of the primer/template duplex was similar to that of template switching assay. Full-length primer extension resulted in a 46-nucleotide-long product similar to that seen in the template-switching assay. Although a number of partial products were observed (as was also the case in the template-switching experiments), only full-length products were used in the analysis. It should be noted that we occasionally observed reduced product formation at the highest substrate concentration (25 μM). Whether this is due to product inhibition or nonspecific substrate binding is not clear.

The concentration of primer extension product formed was calculated and reciprocal plot analysis performed (Figure 2B,C) to determine kinetic values. As Table 1 shows, the catalytic turnover of the DNA polymerase was 28.7 min^{-1}. Recalculated on a per nucleotide basis, the V_{max} for the reaction was 6.1 μM min^{-1}, which results in a catalytic turnover value of 13.8 s^{-1}. This value is in close agreement to previously published reports for the turnover of *E. coli* DNA polymerase I (*6*).

Comparing the kinetic values obtained from the template-switching experiments to those of the primer extension experiment shows that the turnover number for primer extension is about 100-fold higher than for template switching. Furthermore, based on the apparent dissociation constants (K_D^{app}), exo$^-$ KF has a 3.5-fold higher affinity for the primer/template duplex in the primer-extension assay than in the template-switching assay. This, in all likelihood, reflects a structural preference of the exo$^-$ KF for the primer with a continuous template duplex than for the primer/complement duplex with a 3′ overhang.

Non-Template-Directed Nucleotide Addition. As mentioned previously, the template-switching experiments formed a small number of products that were longer than the fully extended products (Figure 2A). These results are consistent with previous results suggesting that non-template-directed nucleotide addition occurred at the end of fully extended products (*11*). The bands were relatively diffuse, suggesting the addition of more than one kind of nucleotide to the ends. Thus, the exact number of nucleotides added is difficult to determine precisely; however, analysis indicated that a range of between 1 and 4 nucleotides were added, with 2 and 3 nucleotide additions (products of between 48 and 49 nucleotides in length) predominating. At later time points (60 min), another group of bands could be observed 2−3 nucleotides longer still, migrating at approximately position 51 to 52 (data not shown), suggesting that nucleotides may be added in a nondistributive manner. It should be emphasized that the non-template-directed nucleotide addition seen occurred after template synapsis and primer extension in the template-switching experiments. Furthermore, little to no non-template-directed nucleotide addition was observed in the primer-extension experiments. These and other observations suggest

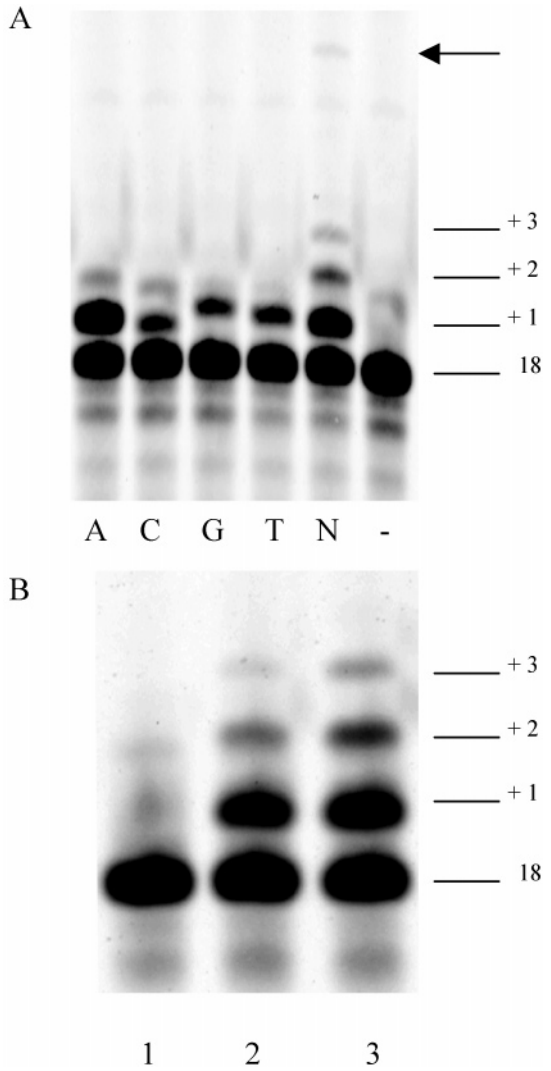

FIGURE 5: Non-template-directed nucleotide addition. (A) The extension products formed from non-template-directed nucleotide addition using the blunt end substrate depicted in Figure 1D at 0.3 mM of the various nucleoside triphosphates (A, dATP; C, dCTP; G, dGTP; T, TTP; N, all four nucleoside triphosphates; "−", in the absence of any nucleoside triphosphates). The substrate is 18 nucleotides long and the number of nucleotides added is denoted on the right. Note that the +n products migrate at different rates due to slight molecular mass and charge differences among the nucleotides. Note also that we consistently observed a +7 product with the complete mix of dNTPs (arrow). (B) Non-template-directed nucleotide addition in the presence of all four nucleoside triphosphates is shown at 0, 15, and 30 min (Lanes 1−3, respectively). The 18-nucleotide-long substrate (*18*) and the three major products (+1, +2, and +3) are denoted.

that the single-stranded template in the template-switching experiments may exert some influence on the length or rate of non-template-directed nucleotide addition (Faucett and Islas, manuscript in preparation).

We were interested in determining the rate of non-template-directed nucleotide addition in the template-switching experiments to compare the magnitudes of the two activities. We therefore quantified the bands that showed only template switching and the bands that showed template switching plus non-template-directed nucleotide addition and determined their respective kinetic values (Table 3). There was no difference between the two for binding affinity to the enzyme; both products showed a K_D^{app} of between 10.7 and 10.9 μM. Furthermore, the V_{max} for the template switching plus primer extension products was slightly lower than that for template switching alone. Since the 48/49-nucleotide-long product is the result of both template switching and non-template-directed nucleotide addition, we reasoned that the turnover number is a reflection of both activities. Therefore, subtracting the time needed for template switching from the time needed for template-switching plus primer extension yielded a $k_{cat} = 0.18$ min^{-1} for non-template-directed nucleotide addition. Extrapolating from the turnover number yielded a $V_{max} = 13$ nM min^{-1}. The V_{max} per nucleotide was approximately 32.5 nM min^{-1} assuming an average event added 2.5 nucleotides.

Table 3: Kinetic Values for Template Switching and Non-Template-Directed Nucleotide Addition

	K_D^{app} (μM) ± SEM	V_{max}^{app} (nM min^{-1}) ± SEM	k_{cat} (min^{-1})	n
template switching + non-template addition (observed)	10.9 ± 0.96	4.68 ± 1.56	0.06	12
template switching only (observed)	10.7 ± 1.83	7.27 ± 1.12	0.10	11
non-template addition only (calculated)		13.0	0.18	

Template switching in these experiments yielded a $k_{cat} = 0.10$ min^{-1}—less that seen in Table 1—due in part to the fact that only a subpopulation of the products were used in this calculation. If all the template-switching products were included, then the catalytic turnover was 0.2 min^{-1}, comparable to the turnover for non-template-directed nucleotide addition and only somewhat lower than that seen in Table 1.

To confirm the non-template-directed nucleotide addition values seen in the template-switching experiments, we tested non-template-directed nucleotide addition activity on a double-stranded blunt substrate (Figure 1D) under saturating substrate and dNTP concentrations (Figure 5). No template switching or primer extension was needed for non-template-directed nucleotide addition to occur in these experiments. The addition of all four nucleotides either individually or in a mixture resulted in non-template-directed nucleotide addition of at least one nucleotide (Figure 5A). Interestingly, dATP and dCTP could add an additional nucleotide (+2 product); no +2 products were seen for dGTP or TTP. Moreover, if all four nucleotides were present, the rate of +2 formation increased and a +3 band appeared. Intriguingly, a small but reproducible band appeared at position +7 and may reflect a small subpopulation of enzymes that add several nucleotides in a processive manner.

Consistent with previous results, exo$^-$ KF prefers dATP—2930 nM product formed after 30 min—to dCTP (412 nM), dGTP (327 nM), and TTP (343 nM). Product formation in the presence of all four nucleotides (1703 nM) for the same period of time was 58% of that for dATP.

We performed a more detailed kinetic analysis on the non-template-directed nucleotide addition activity, examining the kinetic parameters of each nucleoside triphosphate precursor (Table 4). In the presence of all four nucleotides, the determined kinetic values, $V_{max} = 12.1$ nM min^{-1} and $k_{cat} = 0.16$ min^{-1}, were in complete agreement with the values

Table 4: Kinetic Values for Non-Template-Directed Nucleotide Addition with Various dNTP Precursors

	K_D^{app} (μM) ± SEM	V_{max}^{app} (nM min^{-1}) ± SEM	k_{cat} (min^{-1})	k_{cat}/K_D^{app} (μM^{-1} min^{-1})	n
dATP	3.72 ± 1.27	15.8 ± 1.36	0.21	0.058	4
dGTP	12.3 ± 3.96	3.01 ± 0.41	0.04	0.003	4
dCTP	15.6 ± 1.34	1.11 ± 0.12	0.02	0.001	5
TTP	19.2 ± 2.70	2.46 ± 0.35	0.03	0.002	4
dNTPs	7.19 ± 0.51	12.1 ± 0.87	0.16	0.023	4

obtained for non-template-directed nucleotide addition in the template-switching experiments (Table 3). Confirming the results seen in Figure 5A, dATP showed the strongest rates of reaction, with a $V_{max} = 15.8$ nM min^{-1}, slightly higher than that seen with all four nucleotides. The K_m for dATP (3.72 μM) was the strongest of all four nucleotides and nearly half of that seen with all four nucleotides. Therefore, dATP has a 3.3−5.2-fold better binding affinity and a 5.2−14-fold higher turnover number over the other three nucleotides, resulting in a 20−50 higher catalytic efficiency. The K_m in the presence of all four nucleotides is nearly double that of dATP, even though the V_{max} is roughly comparable, suggesting that the presence of the other nucleotides may competitively inhibit non-template-directed nucleotide addition. Note that the determined K_D^{app} values for each nucleotide (with the exception of dGTP) in the presence of the duplex substrate was approximately 2−3-fold stronger than that previously reported for either Pol I or KF (6): K_D for both purine nucleotides is approximately 8−10 μM and the K_D for pyrimidine nucleotides is approximately 45−60 μM. Whether this 2−3-fold difference is the result of the way the values were experimentally determined is certainly a possibility. However, the dGTP binding affinity determined here was weaker than that for dATP and approaches the binding affinities seen for pyrimidine nucleotides. The implication is that exo$^-$ KF discriminates between dATP and dGTP to a greater extent in the presence of blunt-ended substrates than under normal primer extension conditions, and may also reflect a similar preference as that seen in the presence of abasic sites (the "A rule").

Taken together, these data suggest that most, if not all, of the non-template-directed nucleotide addition is the result of dAMP incorporation to the primer strand. This would predict that for blunt-end substrates, one or more deoxyadenosine residues are added, and synapsis to a template strand containing thymidine residues at its end would be more efficient than any other sequence. Recent experiments in our lab suggest that this is indeed the case (Faucett and Islas, manuscript in preparation).

DISCUSSION

This study was designed to determine the magnitude for the rates of non-template-directed nucleotide addition and template switching by exo$^-$ KF, to understand how these activities affect DNA replication and mutagenesis. Furthermore, the study was designed to examine the mechanism and binding kinetics of two discontinuous DNA templates to DNA polymerase.

We quantitatively show that the rates of template switching and non-template-directed nucleotide addition by exo$^-$ KF to be comparable in magnitude to one another and to be approximately 2 orders of magnitude slower than normal template-directed DNA synthesis. In other words, in the absence of proofreading activity and under saturating concentration of free-ends, DNA polymerase I will add nucleotides at DNA ends and switch templates at the same rate it synthesizes approximately 3 kb of DNA. Although the relative magnitudes of these activities are small—the turnover for non-template-directed nucleotide addition was determined to be 0.18 min^{-1} and the turnover for template switching was determined to be 0.26 min^{-1}—they are comparable to the magnitude of the 3′-5′-exonuclease activity reported for *E. coli* DNA polymerase I (*6*). Furthermore, these data suggest that when exonuclease-free DNA polymerases encounter discontinuities in their template strands they can synthesize short stretches (1−7 nucleotides) of DNA in a non-template-directed manner, and use these overhangs to anneal an unlinked template and continue DNA synthesis. The kinetic analysis presented for non-templated-directed nucleotide addition suggests that nearly all of the nucleotides added to the end of DNA are deoxyadenosine residues.

Kinetic results varying both substrates suggest that exo$^-$ KF binds the two discontinuous substrates in a random sequential order and that each substrate partially inhibits the binding of the other. These results clearly indicate that binding of two discontinuous DNA molecules is not only possible, but also relatively common. It should be pointed out that the catalytic turnover for template switching is a composite of template synapsis and primer extension. Therefore, it is possible that the lower catalytic turnover seen may also reflect a slower primer extension rate than that seen for normal primer extension. More likely though, the lower turnover is a reflection of relatively small number of enzyme/P:C-substrate/T-substrate complexes at any given time (even at saturating substrate concentrations) due in part to the mixed-type inhibition by each substrate.

In the template-switching studies described here, a three-nucleotide-long overhang was used to mimic a non-template-directed event. The overhang consisted of a 5′-ATA-3′ sequence. The theoretical T_m for this sequence is approximately 6 °C (assuming 2 °C per A:T base pair)—well below the reaction temperature—suggesting that if any hybridization between the two substrates occurs, they quickly denature. Moreover, with these sequences, an overhang would have to be between 12 and 14 nucleotides to be thermally stable ($T_m \sim 37$ °C); at that point primer extension of spontaneously hybridized substrates would be a competing reaction. Our results for non-template-directed nucleotide addition suggest that the longest overhang created was seven nucleotides ($T_m \sim 16$ °C) but with most of the nontemplated events occurring between 1 and 3 nucleotides in length, which are too short for the hybridization products to be thermally stable. Interestingly, a 3−4-nucleotide-long overhang appears to be optimal for template switching for exo$^-$ KF and human DNA polymerase α (*11*, *12*), again suggesting that the non-template-directed nucleotide addition is an important activity for template switching.

Exo$^-$ KF was used in these studies because it shows among the strongest template-switching activity of any DNA polymerase examined thus far, and as such it serves as a model for other proofreading-deficient enzymes, most notably DNA polymerase α and other eukaryotic polymerases. Note that exonuclease-proficient enzymes also have small

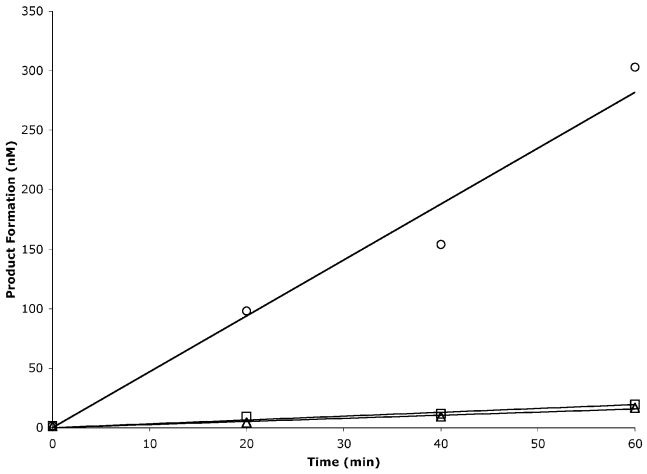

FIGURE 6: The concentration of products formed as a function of time by the template-switching activities of three forms of DNA polymerase I. Reactions were performed under saturating substrate concentrations. Exo$^-$ KF, (open circles); KF (open triangles); DNA polymerase I (open squares).

but significant template-switching activity. In our hands, KF and DNA polymerase I also show template-switching activity (Figure 6)—at levels about 10−15-fold lower than that seen for exo$^-$ KF—suggesting that although the absence of proofreading activity is important, it is not sufficient to prevent template switching (see below).

Is there any in vivo evidence to support this model? The strongest in vivo evidence for the role of non-template-directed DNA synthesis comes from analyses of plasmid recircularization in mammalian cells. Roth et al. observed that nearly 10% (18 out of 200) of junctions in nonlymphoid cells showed non-template-directed insertions consistent with the idea that DNA polymerase adds nucleotides to the ends of the plasmid in a non-template-directed manner (*15*). Furthermore, in TdT −/− mouse cells, 3% of junctions resulting from V(D)J recombination showed non-template-directed nucleotide insertions (N-nucleotides) of between 1 and 3 nucleotides in length (*16*). Since TdT is absent, the non-template-directed nucleotide addition is most likely due to other endogenous DNA polymerases. This frequency is consistent with the magnitude we see for non-template-directed nucleotide addition in this study and suggests that both eukaryotic and prokaryotic DNA polymerases—mostly likely those missing proofreading activity—carry out non-template-directed nucleotide addition at DNA ends.

The in vivo significance of template switching is more difficult to establish, although evidence for the type of template switching proposed by Kornberg and his colleagues has been reported in *E. coli* (*17*). A number of spontaneous multiple base substitutions occur at pseudo-inverted repeats (i.e., quasi-palindromes), suggesting that replicative DNA polymerases can perform template switching in two ways: through interstrand switching from the template to the displaced parental strand or through intrastrand template switching from the template to the primer strand, involving a hairpin intermediate (*18*−*21*). Despite the relatively low spontaneous mutation frequency seen, these results clearly indicate that DNA polymerase can release one template strand and bind another during replication. How interaction between DNA polymerase and the pseudo-inverted repeats, or other structural features in the DNA (e.g., damage), contributes to template switching is still not clear.

Template switching, defined here as DNA synthesis resulting from the juxtaposition of two physically discontinuous template strands, may be a more general phenomenon than the mutagenic events seen at quasi-palindromes. For example, this may well explain the phenomenon of oligonucleotide capture (*22*) (Faucett and Islas, manuscript in preparation). Moreover, strong evidence exists that the reinitiation of stalled replication forks as a result of DNA damage on one of the strands may result in the regression of the fork and the creation of a so-called "chicken-foot" intermediate. The blocked DNA polymerase on the damaged template uses the nascent sister chromatid as a template to synthesize DNA past the replication block, in essence switching templates (*23*). This event in *E. coli* clearly appears to be recA dependent. Factors involved in homologous recombination may play an important role in primer-template synapsis. However, it is possible that on rare occasions DNA polymerase alone may perform this same sort of template-switching activity. Indeed, the recombination-dependent structures recently proposed for the reinitiation of stalled replication forks as well as those proposed by Higgins et al. nearly 30 years ago (*24*), show some of the same general features to the model that Kornberg and colleagues proposed to describe the template-switching activity in *E. coli* DNA polymerase I (*7*). An important difference is that in the latter case DNA polymerase switches templates to the displaced complementary parental strand, presumably near a break in that strand. Whether recombination factors act to inhibit DNA polymerase-mediated template switching and promote recombinational repair is still not clear. Whether DNA polymerase is present during synaptonemal formation in recombinational repair also remains unclear.

With as many as five prokaryotic DNA polymerases and 16 eukaryotic DNA polymerases, it is tempting to speculate that many of the so-called translesion bypass polymerases may also carry out non-template-directed nucleotide addition and template switching as a possible mechanism for nonhomologous end joining. In fact, DNA polymerase λ, a DNA polymerase β homologue and member of the X family of DNA polymerases, has been implicated in gap filling of mismatched DNA during end joining (*25*). Furthermore, DNA polymerase β has been shown to carry out template switching in vitro at relatively high enzyme concentrations (Islas, unpublished observations). Exo$^-$ KF carries out template switching in vitro although wild-type KF does so to a much lesser degree (Figure 6), suggesting that the 3′-5′-exonuclease activity may remove the 3′ overhangs and thus severely reduce template-switching activity. Since in some of these translesion DNA polymerases (e.g., DNA polymerase η, ι, λ, μ in eukaryotes and DNA polymerase IV and V in *E. coli*) no 3′-5′ exonuclease activity has yet been identified (*26*), it is possible that non-template-directed nucleotide addition by these polymerases may facilitate nonhomologous end joining through the type of template-switching mechanism we propose here.

Because of the potentially mutagenic effects of template switching and non-template-directed nucleotide addition, these activities may be regulated or inhibited in vivo. For example, non-template-directed nucleotide addition by terminal deoxynucleotidyl transferase (TdT) is regulated by the

nonhomologous end-joining factor Ku (*27*). Furthermore, TdT occurs in two isoforms: a short isoform and a long splice variant. The latter contains a 3′-5′ exonuclease domain which results in a coordinated balance between non-template-directed nucleotide addition and nucleolytic removal at coding joints of antigen receptor genes during V(D)J recombination (*28*). These data suggest that non-template-directed nucleotide addition must be regulated to avoid potentially mutagenic events at strand breaks. It is still an open question how non-template-directed nucleotide addition and template switching demonstrated by exo$^-$ KF or by human DNA polymerase α are regulated in vivo. The data presented here strongly suggest that the magnitude of these activities may be sufficiently robust over the lifespan of a cell to incur a significant mutagenic load.

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